# US Imaging of Tumor Angiogenesis with Microbubbles Targeted to Vascular Endothelial Growth Factor Receptor Type 2 in Mice

## Appendix E1

The following sections describe the principle of targeted contrast-enhanced US imaging in more detail.

### *General Approach for Image Processing*

For image processing, two data sets are used: a predestruction data set and a postdestruction reference data set. These data sets typically refer to a series of sequential image frames composed as a cine clip. During acquisition of the two data sets, the position of the animal must be as stable as possible to prevent errors in measurements. Small changes in image positioning due to respiration and cardiac motion are taken into account by using multiple frames as opposed to a single frame for image processing. Each frame in the predestruction data set is compared with each frame in the postdestruction reference data set. The best match is then used in the subtraction process (see below). The best match indicates the highest spatial correlation between the data from the predestruction data sets and a frame from the postdestruction reference data set. These two correlated frames are subtracted, and the resulting difference in video intensity represents the change in video intensity due to the presence of adherent microbubbles (see below). This difference is displayed by the software as a colored (green) overlay on the grayscale images.

### *Cine Loop of Predestruction Data Sets*

This cine loop is acquired before destruction of the microbubbles and is a set of n images. In our study, the cine loop was acquired 4 minutes after intravenous administration of the microbubbles and consisted of 120 frames. After 4 minutes (to allow the targeted microbubbles to float into the tumor vessels and to bind to the VEGFR2 on the endothelial cells of the tumor vessels), the video intensity is composed of three components: *(a)* the videointensity from the tumor tissue, *(b)* the video intensity from microbubbles that are not attached to the receptors (circulating microbubbles), and *(c)* the video intensity from microbubbles attached to the receptors on the endothelial cells (Fig 1c).

### *Cine Loop of Postdestruction Reference Data Sets*

The cine loop of postdestruction reference data sets is a set of n images acquired after destruction of the targeted microbubbles (the microbubbles are destroyed in real time by applying a high-power pulse through the transducer). This cine loop of reference data sets should be long enough to encompass two to three respiration cycles and two to three cardiac cycles. At a frame rate of 20 Hz, we chose 120 frames for the purpose of the study. After destruction of the microbubbles in the imaging plane and replenishment of the vessels by nonattached, freely circulating microbubbles, the video intensity consists of two components: *(a)* the video intensity from the

tumor tissue and *(b)* the video intensity from microbubbles that are still circulating in the vessels (Fig 1c).

***Pairing of Pre- and Postdestruction Cine Loops and Subtraction of Data Sets***

First, images from both the pre- and postdestruction cine loops are paired. Since there are small positional changes of the animal due to respiratory and cardiac movements, as well as slight motion of the animal on the table, the pre- and postdestruction data sets are compared by using an absolute-sum-of-differences technique, with pairing of the two images with the smallest total difference (or smallest net error) (Eq E1). This means that the two frames with the smallest total difference are the most similar in anatomic composition.

$$\text{Net Error} = \sum_{i}^{\text{All Pixels}} (\text{Reference Image}[i] - \text{DataImage}[i])^2 \qquad \text{(E1)}$$

After pairing the frames of the two cine loops, the data sets are subtracted and the differences are displayed as a semitransparent green overlay on the grayscale images by use of a blending algorithm. The level of transparency is controlled by the user.